(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 307 158
A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 88308213.3

(22) Date of filing: 05.09.88

(51) Int. Cl.4: C08B 37/00 , C12P 19/18 , A61K 31/70 , A21D 2/18 , //C12N9/10,C12R1:66

(30) Priority: 04.09.87 JP 221347/87

(43) Date of publication of application: 15.03.89 Bulletin 89/11

(84) Designated Contracting States: AT BE CH DE ES FR GB GR IT LI LU NL SE

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(54) Process for the preparation of branched fructooligosaccharides.

(57) This invention relates to a method for production on an industrial scale of the branched fructooligosaccharide indicated by the general structural formula below using microorganisms or an enzyme produced by microorganisms which belong to the genus Aspergillus.

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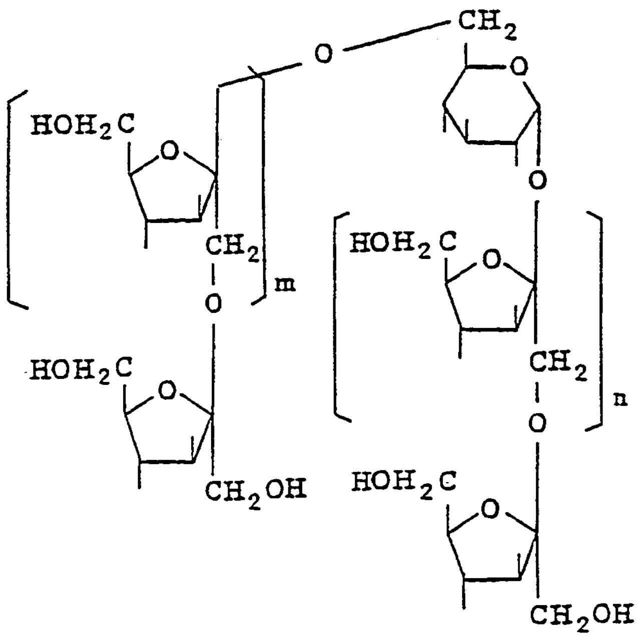


(In the formula above, m=0-10, n=0-8 and $3 \leqq m+n \leqq 12$.)

Process for the preparation of Branched Fructooligosaccharides

This invention relates to a method, utilizing microorganisms or an enzyme which is produced by microorganisms belonging to the genus Aspergillus, for the production of branched fructooligosaccharides having a specific structure, using sucrose as raw material.

The physiological activity possessed by fructooligosaccharides has recently become of major interest ["Kagaku to Seibutsu" (Chemistry and Biology), Vol. 21, p. 291]. For example, fructooligosaccharides are difficult to digest and are selectively utilized by useful intestinal flora, Lactobacillus bifidus in particular, thereby promoting proliferation of these organisms and improving laxation and the like. In addition, when fructooligosaccharides are broken down by Lactobacillus bifidus, organic acids are produced. These have been recognized to have the effect of reducing cholesterol levels in the body.

Fructooligosaccharides are formed by the action of a fructose transferase (fructosyl transferase) on sucrose. Microorganisms that are known to form fructose transferases include yeast, Aspergillus niger, Aureobasidium pullulans, etc. In addition, as has been previously shown by the inventors, fructooligosaccharides can also be effectively produced using fructose transferase produced by Aspergillus sydowi [see TOKKYO-KOKAI-KOHO (18-month Publication of Unexamined Patent Application) SHOWA 61(1986)-187797 (hereinafter referred to as TOKKAISHO 61-187797)].

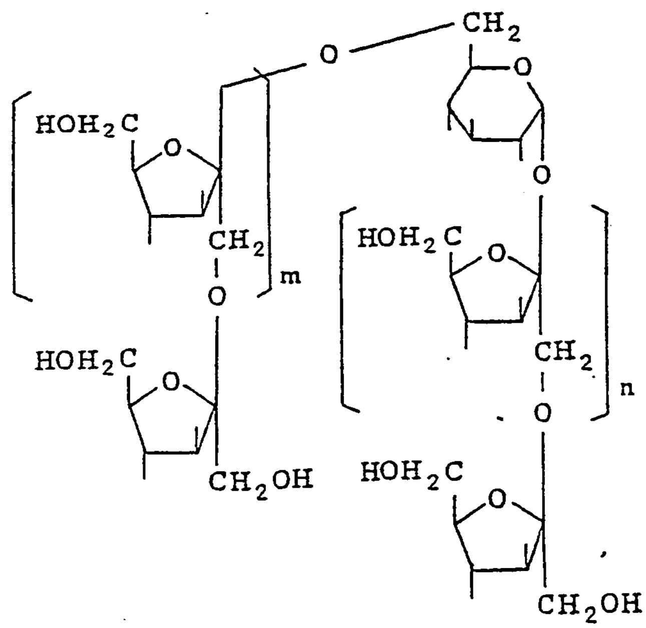
However, these fructose transferases acted on sucrose and either formed fructooligosaccharides with a structure in which several fructose are linked by β-1,2 bonds to the fructose residue of sucrose, or formed high molecular weight polyfructan composed of the same linkages. Examples of sugars obtained using the fructose transferase produced by microorganisms such as those indicated above and having structures other than these have thus far not been reported.

Conversely, it is known that different fructooligosaccharides are produced by various plants ("Kagaku to Seibutsu", Vol. 18, p. 674). It has been reported that non-reducing fructose polymers having a degree of polymerization of 3-15 exist in the storage roots of asparagus in particular, and their structures have been elucidated [N. Shiomi, J. Yamada & M. Izawa, Agric. Biol. Chem., 40, 567 (1976), 43, 1375 (1979), 43, 2233 (1979)]. The fructooligosaccharides found in the storage roots of asparagus have a structure in which fructose is linked at both the glucose residue and fructose residue of sucrose resulting in the structure having a branching form. Fructooligosaccharides having these structures were not found in fructooligosaccharides that are formed using microorganisms.

The purpose of this invention is to provide a method for the production of branched fructooligosaccharides so that fructooligosaccharides substantially identical to naturally-occurring branched fructooligosaccharides, which have heretofore been known only to exist in higher plants, are able to be industrially produced using microbial enzymes.

As was previously stated, although the inventors had previously shown that fructooligosaccharides such as 1-ketose and nystose, in which fructose is linked by -1,2 bonds at the fructose residue of sucrose, can be obtained utilizing the mycelia of Aspergillus sydowi, as a result of subsequent research, it has now been found that branched fructooligosaccharides in which fructose is linked to both the glucose residues and fructose residues of sucrose can be obtained simultaneously with the above fructooligosaccharides under the same reaction conditions.

In summary, the present invention provides a process for the preparation of branched fructooligosaccharides of formula A.



······ (A)

(wherein m = 0-11, n = 0-8, and $3 \leq m + n \leq 12$) said process comprising the step of treating a sucrose solution with the mycelium of a fructose-transferase producing microorganism of the genus Aspergillus, or with a fructose-transferase enzyme prepared from said organism.

Although any member of the genus Aspergillus which has the ability to produce fructose transferase would be satisfactory as the microorganism used in this invention, Aspergillus sydowi is particularly preferable. Examples of stock types of Aspergillus sydowi used include IAM 2544, IAM 2514, IAM 2078 and IAM 2009 (all of which are Type Culture Collection Numbers of Institute of Applied Microbiology, University of Tokyo).

According to this invention, it is possible, using the mycelia of microorganisms or the enzyme prepared from such mycelia to produce on an industrial scale branched fructooligosaccharides, which had heretofore only been known in higher plants such as the storage roots of asparagus. In addition, since the branched fructooligosaccharides that are obtained have a structure that is substantially identical to the naturally-occurring branched fructooligosaccharides mentioned above, they have a high level of safety when added to food products. Furthermore, these branched fructooligosaccharides are expected to demonstrate various effects in terms of their physiological activity, including the promotion of proliferation of Lactobacillus bifidus in the intestines of humans, thereby improving laxation.

Although either a solid or liquid medium may be used as the growth medium for Aspergillus sydowi, the microorganism used in this invention, in the case of a liquid medium, a medium containing the following components is particularly preferred.

Glucose 10% (W/V)
Corn Steep Liquor 2%
$MgSO_4 \cdot 7H_2O$ 0.1%
KH_2PO_4 0.2%
pH = 6.0

Aspergillus sydowi is inoculated into the medium that is described above and incubated under aerobic conditions by, for example, a shake culture using a rotary shaker, etc., or an agitation aeration culture using a jar fermentor, etc. An incubation temperature of approximately 30° C is suitable.

When the organism is incubated for several days under these conditions, large numbers of mycelia of Aspergillus sydowi are formed and incubation is concluded at this point. The mycelia are then collected by means of centrifugation and filtration. After washing with physiological saline, the mycelia are preserved using lyophilization. Furthermore, in the case of using a microorganism that belongs to the genus Aspergillus other than Aspergillus sydowi, the mycelia of that microorganism can be obtained using a similar method to that described above.

In this invention, branched fructooligosaccharides are produced by treating sucrose using the mycelia

of, or enzymes prepared from the mycelia of, a microorganism which has the ability to produce fructose transferase and belongs to the genus Aspergillus, the mycelia being obtained using the method described above.

Although the mycelia can be used in their natural form, it is also possible to use mycelia that have been immobolised, for example in alginate, acrylamide gel, polyvinyl alcohol gel, photo cross-linking resin, carrageenan, chitosan or gelatin. Furthermore, the mycelia may also be treated with glutaraldehyde and the like in order to increase carrier strength. When non-immobilised mycelia are used it is necessary to carry out the sucrose treatment using batch methods, however, immobilised mycelia can be charged on a column and continuously fed, allowing the reaction to be performed more efficiently than by batch methods. In addition, when enzyme that has been prepared from the mycelia is to be used, the mycelia are homogenised and an enzyme solution is extracted. This enzyme solution can then be used, further purifying as necessary. In addition, the enzyme that is obtained can also be used immobilized onto a suitable carrier.

A pH of 5.0-7.0 is desirable and a pH of 5.5-6.5 is optimum for enzyme reaction conditions for the formation of branched fructooligosaccharides. A temperature of 30-70° C is desirable and a temperature of 40-60° C is even more desirable for the temperature conditions. The concentration of sucrose which is used for the raw material should be 30-80% (W/V), more preferably 50-80% (W/V). In addition, the amount of fructose transferase that is used at that time should be 5 units or more per 1g of solid sucrose. 1 unit here is defined as the amount of enzyme which will transfer 1µmol of the fructose residue of sucrose to another sucrose molecule or to a branched fructooligosaccharide in 1 minute at pH 6.0 and 60° C at a substrate concentration of 50% (W/V) sucrose in solution.

After filtration, using for example, a membrane filter, deionization and decolorization of the reaction solution thus obtained, the solution can be concentrated and made into a syrup or made into a powder by spray drying. Furthermore, although the reaction solution contains a total of 30-50% (W/W) glucose, fructose and unreacted sucrose in addition to the fructooligosaccharides, these can be removed by either gel filtration using "Bio-Gel" (trademark; mfd. by Bio-Rad Co., Ltd.) or "Toyopearl HW40" (trademark; mfd. by Toyo Soda Kogyo Co., Ltd.), or by strongly acidic cation exchange resin column chromatography. It is also possible to obtain only branched fructooligosaccharide in high purity by separating the branched fructooligosaccharide from fructooligosaccharides such as 1-ketose and nystose using a method similar to that described above.

As was previously indicated in the general structural formula (A), the branched fructooligosaccharide obtained by this invention is an oligosaccharide with a degree of polymerization of 6 or more with 1-11 fructose moieties linked to the glucose residue of a sucrose molecule and 1-9 fructose moieties linked to the fructose residue. This branched fructooligosaccharide has a structure that is substantially the same as that of naturally-occurring fructooligosaccharide contained in the storage roots of asparagus and has an extremely high degree of safety, with regard to its use as a food additive.

In the same manner as conventional fructooligosaccharides, the branched fructooligosaccharide obtained with this invention is expected to have the effect of action to promote proliferation of Lactobacillus bifidus in the intestines of humans, thereby improving laxation. In addition, since results of blood sugar loading tests have shown that, not being hydrolyzed by digestive enzymes in the body, the fructooligosaccharides do not raise blood sugar levels, applications for diabetic patients have also been considered. Furthermore, the branched fructooligosaccharides have also been recognized to act in reducing cholesterol and neutral fat levels in the blood and liver.

Product forms of the branched fructooligosaccharide obtained with this invention include health food products in the form of a powder prepared by spray drying and a concentrated liquid, as well as use as an additive in other foods such as bread and biscuits.

Example 1

100ml of the liquid medium indicated below (pH 6.0) was placed in a Sakaguchi flask and Aspergillus sydowi IAM 2544 (Type Culture Collection Number of Institute of Applied Microbiology, University of Tokyo) was inoculated to the medium from a slant. This was then cultivated with reciprocal shaker for 5 days at 30° C. The organisms were collected by centrifuging the culture liquid and after washing several times with physiological saline, were lyophilized and submitted for testing.

Glucose 10% (W/V)
Corn Steep Liquor 2%
$MgSO_4 \cdot 7H_2O$ 0.1%
KH_2PO_4 0.2%

pH = 6.0

Next, 5 units of the above mycelia were added per 1g of solid sucrose to a 50% (W/V) sucrose solution (pH 6.0). While stirring at a temperature of 50 °C, the solution was allowed to react for 2 days. Continuing, after removing the mycelia by filtration, each of the fructooligosaccharides was separated into fractions by carbon column chromatography. These fractions were then purified by gel filtration chromatography using "Toyopearl HW40S" (trademark; mfd. by Toyo Soda Kogyo Co., Ltd.) to obtain fructooligosaccharides of high purity that possess a series of degrees of polymerization.

The series of fructooligosaccharides that were thus obtained were hydrolyzed using 0.1N hydrochloric acid or invertase to determine the respective molar ratios of glucose and fructose. The results of this are shown in Table 1.

Table 1

(Molar Ratios of Glucose and Fructose Following Hydrolysis of Formed Fructooligosaccharides)				
	HCl Hydrolysis		Invertase Hydrolysis	
	Glucose	Fructose	Glucose	Fructose
GF_2	1.0	2.0	1.0	2.2
GF_3	1.0	3.0	1.0	3.0
GF_4	1.0	3.9	1.0	3.9
GF_5	1.0	5.2	1.0	5.0
GF_6	1.0	6.3	1.0	6.3
GF_7	1.0	7.6	1.0	7.2
GF_{8-12}	1.0	8.6	1.0	8.2
(In the table, G refers to glucose and F refers to fructose.)				

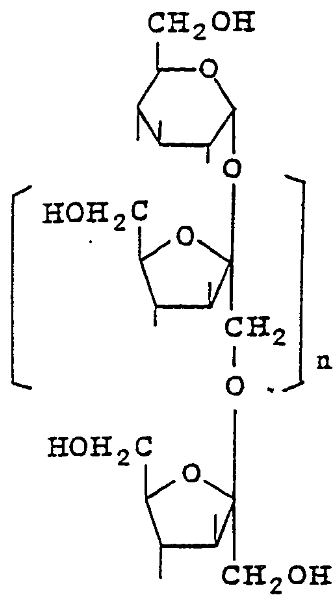
As is indicated in Table 1, the rise in the molar ratio of fructose accompanied the increase in the degree of polymerization irregardless of which method of hydrolysis was employed. It has therefore been estimated that the structure of these fructooligosaccharides is such that fructose is linked to sucrose by β-fructofuranoside linkages.

Continuing, after methylation of these fructooligosaccharides by the known method of Hakomori, they were hydrolyzed with acid followed by reduction to glucitol acetates. These glucitol acetates were then analyzed by capillary gas chromatography. The results of this analysis are shown in Table 2.

Table 2

(Molar Ratios of Permethylated Glucitol Acetates)					
	2,3,4,6-Tetramethyl	2,3,4-Trimethyl	1,3,4,6-Tetramethyl	3,4,6-Trimethyl	3,4-Dimethyl
GF_2	1.0	-	1.0	0.9	-
GF_3	1.0	-	1.0	2.2	-
GF_4	1.0	-	1.0	3.3	-
GF_5	-	1.0	1.9	3.2	-
GF_6	-	1.0	1.7	4.0	-
GF_7	-	1.0	2.0	6.6	-
GF_{8-12}	-	1.0	1.8	6.9	-

As is indicated in Table 2, for the ratios of the peak areas of the permethylated sugars that were obtained, in contrast to the ratio of 2,3,4,6-TMG : 1,3,4,6-TMG : 3,4,6-TMG being 1 : 1 : n-1 for $GF_{n=2-4}$, the ratio of 2,3,4-TMG : 1,3,4,6-TMG : 3,4,6-TMG was 1 : 2 : n-2 for $GF_{n=5-12}$. Based on this, it was clear that from among the fructooligosaccharides that were obtained, $GF_{n=2-4}$ were fructooligosaccharides that have the structure indicated by the general structural formula (B) below, and $GF_{n=5-12}$ were branched fructooligosaccharides that have the structure indicated by the general structural formula (A) which was shown earlier.



...... (B)

(In the formula above, n = 1-3.)

As has been shown thus far, it is clear that the fructooligosaccharides with a degree of polymerization of 6 or greater that were obtained by allowing the mycelia of Aspergillus sydowi to act on sucrose are new branched fructooligosaccharides. Although these branched fructooligosaccharides are those found in the storage roots of asparagus, one of higher plants, these are the first to have been found as fructooligosaccharides that were produced by microorganisms.

Example 2

In this example, a study was made of the effects of substrate concentration, added amount of mycelia and reaction temperature on the production of branched fructooligosaccharides using the lyophilized mycelia obtained in Example 1. The results of these studies are shown in Tables 3 through 5. As can be seen in the tables, a concentration of sucrose, the substrate, of 30% (W/V) or greater is preferable. In regard to the amount of mycelia enzyme that is added, a minimum of 5 units per 1g of solid sucrose is preferable. In addition, it was also determined that a reaction temperature of 40-60 °C yielded favorable results.

Table 3

(The Effect of Sucrose Concentration on Fructooligosaccharide Formation)					
Sucrose Conc. in Substrate Soln. (%)	Formed Sugar Composition (%)				
	GF_{5-12}	GF_{2-4}	Sucrose	Glucose	Fructose
1	-	5.2	40.5	31.2	19.3
5	1.9	19.1	13.3	40.1	23.8
10	1.9	25.5	14.2	36.8	21.5
30	7.2	33.7	8.6	38.3	12.0
50	10.9	30.9	12.3	37.2	8.2

Reaction Conditions: Mycelia having 5 units per 1g of solid sucrose of transferase activity were reacted with various concentrations of sucrose solutions (pH 6.0) at 50° C for 88 hours.

GF_{5-12}: Refers to branched fructooligosaccharides (with general structural formula A) having 4-11 fructose linked to sucrose.

GF_{2-4}: Refers to fructooligosaccharides (with general structural formula B) having 1-3 fructose linked to sucrose.

Table 4

(The Effect of Amount of Added Mycelia Enzyme on Fructooligosaccharide Formation)					
Amt. of Mycelia Enzyme Added to Sucrose Soln. (unit/g)	Formed Sugar Comoposition (%)				
	GF_{5-12}	GF_{2-4}	Sucrose	Glucose	Fructose
1	2.0	33.7	38.1	22.4	3.7
2	4.5	40.9	20.0	30.0	4.2
5	10.9	30.9	12.2	37.2	8.2
10	11.0	30.0	12.1	38.0	8.5

Reaction Conditions: Various concentrations of mycelia enzyme (fructose transferase) were reacted with 50% sucrose solution (pH 6.0) at 60° C for 48 hours.

Table 5 (The Effect of Reaction Temperature on Fructo-oligosaccharide Formation)

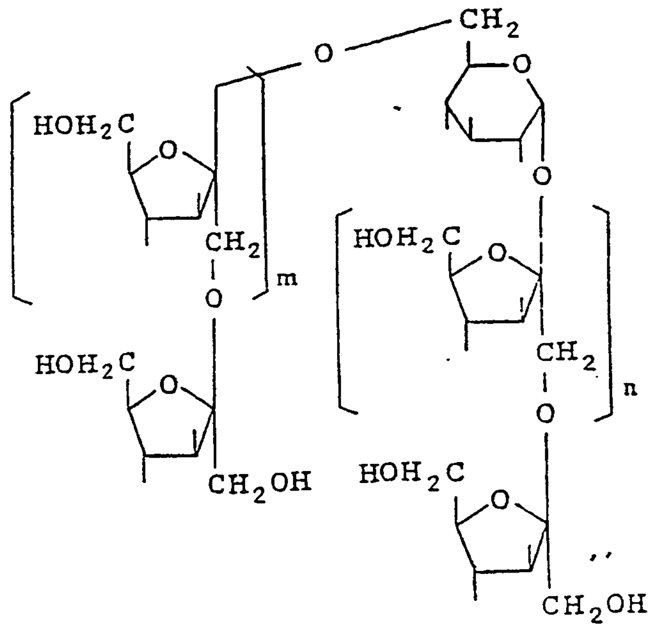
Reaction Temp. (^{o}C)	Formed Sugar Composition (%)				
	GF_{5-12}	GF_{2-4}	Sucrose	Glucose	Fructose
30	4.1	30.4	28.8	29.3	7.3
40	7.5	33.6	18.6	33.3	6.8
50	10.9	30.9	12.3	37.2	8.2
55	8.6	35.4	9.7	35.9	10.3
60	7.2	35.5	8.4	35.7	12.8
70	5.4	34.4	7.3	37.4	15.4

Reaction Conditions:

Mycelia having 5 units of fructose transferase enzyme activity per 1g of solid sucrose were reacted with 50% sucrose solution (pH 6.0) at various temperature conditions for 88 hours.

Claims

1. A process for the preparation of branched fructooligosaccharides of formula A



(A)

(wherein M=0-11, n=0-8, and 3 ≤ m+n ≤ 12)
said process comprising the step of treating a sucrose solution with the mycelium of a fructose-transferase producing microorganism of the genus Aspergillus, or with a fructose-transferase enzyme prepared from said organism.

2. A process as claimed in claim 1 wherein said microorganism is Aspergillus sydowi.

3. A process as claimed in claim 1 or claim 2 wherein said microorganism or said enzyme is in immobilised form.

4. A process as claimed in any one of claims 1 to 5 wherein said fructose transferase enzyme is supplied at a concentration of at least 5 units per gram of sucrose.

5. A process as claimed in any one of claims 1 to 6 comprising the further step(s) of subjecting the resulting reaction solution to at least one of the following treatments: filtration, deionisation, decolorisation and concentration.

6. A process as claimed in any one of claims 1 to 5 comprising the further step of purifying branched fructooligasaccharide from the resulting reaction solution using gel filtration or ion exchange chromatography.

7. A process as claimed in anyone of claims 1 to 6 comprising the further step of separating branched fructooligoccharides of formula A, from other fructooligosaccharides.

8. A compound of formula A as defined in claim 1 substantially free of contaminants of plant origin.

9. Use of a branched fructooligasaccharide of formula A as defined in claim 1 for the preparation of a composition for use in improving laxation.

10. Use of a branched fructooligasaccharide of formula A as defined in claim 1 for the preparation of a food additive.

11. A food additive comprising as active ingredient a branched fructooligosaccharide of formula A as defined in claim 1.

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(30) Priority: 04.09.87 JP 221347/87

(43) Date of publication of application:
15.03.89 Bulletin 89/11

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(88) Date of deferred publication of search report:
02.11.89 Bulletin 89/44

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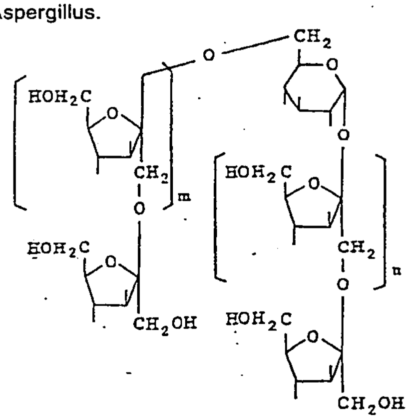

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EP 0 307 158 A3

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EUROPEAN SEARCH REPORT

Application Number

EP 88 30 8213

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
P,X	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 52, no. 5, May 1988, pages 1303-1304, The Agricultural Chemical Society of Japan; M. MURAMATSU et al.: "Structures of some fructooligosaccharides produced from sucrose by mycelia of aspergillus sydowi IAM 2544" * Whole article *	1-8	C 08 B 37/00 C 12 P 19/18 A 61 K 31/70 A 21 D 2/18 C 12 N 9/10 // C 12 R 1/66
Y	PATENT ABSTRACTS OF JAPAN, vol. 11, no. 132 (C-417)[2579], 24th April 1987, page 157 C 417; & 61 268 190 (MEIJI SEIKA KAISHA LTD) 27-11-1986 * Abstract *	1-11	
D,A	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 43, no. 6, 1979, pages 1375-1377; N. SHIOMI et al.: "A novel pentasaccharide in the roots of asparagus"		TECHNICAL FIELDS SEARCHED (Int. Cl.4)
Y	PATENT ABSTRACTS OF JAPAN, vol. 8, no. 41 (C-211)[1478], 22nd February 1984, page 123 C 211; & JP-A-58 201 980 (MEIJI SEIKA K.K.) 25-11-983 * Abstract *	1-11	C 12 P C 08 B

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	08-08-1989	LENSEN H.W.M.

CATEGORY OF CITED DOCUMENTS

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